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Growth performance, nutrient digestibility and intestinal morphology of rainbow trout (*Oncorhynchus mykiss)* fed graded levels of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus*

Aleksandar Vidakovic*[1], David Huyben[1], Henrik Sundh[2], Andreas Nyman[1], Jouni Vielma[3], Volkmar Passoth[4], Anders Kiessling[1], Torbjörn Lundh[1]

[1]Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, P.O. Box 7024, 750 07 Uppsala, Sweden.

[2]SWEMARC, Fish Endocrinology Laboratory, Department of Biological and Environmental Sciences, University of Gothenburg, P.O. Box 463, 405 30 Gothenburg, Sweden

[3]Natural Resources Institute Finland (Luke), Survontie 9 A, 405 00 Jyväskylä, Finland.

[4]Department of Molecular Sciences, Uppsala BioCenter, Swedish University of Agricultural Sciences, P.O. Box 7015, 750 07 Uppsala, Sweden

Corresponding author: *Aleksandar Vidakovic; telephone: +46 (0) 072 247 44 02, E-mail address: Aleksandar.vidakovic@slu.se

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Abstract

In a 10-week study, we evaluated the effects of replacing 20%, 40% or 60% of fishmeal (present in control diet at 300 g kg^{-1}) on a digestible protein basis with yeast *Saccharomyces cerevisiae* or a yeast mixture of *Wickerhamomyces anomalus* and *S. cerevisiae* on growth performance, nutrient digestibility, nutrient retention and intestinal health of rainbow trout (*Oncorhynchus mykiss*). Triplicate tanks with 35 rainbow trout (144.7 ± 25.1 g mean ± SEM) were fed rations of 1.5% of total biomass per tank. Replacement of 60% of fish meal with yeast mixture resulted in lower specific growth rate of 1.0 versus 1.2% day^{-1} for other diets. Apparent digestibility coefficients for crude protein and most amino acids were highest in fish fed fish meal-based diet, with similar values for fish fed the diet with 20% replacement with yeast mixture. Diet with 20% replacement with yeast mixture resulted in highest phosphorus digestibility. Replacement of 60% of fishmeal with *S. cerevisiae* resulted in oedematous mucosal fold tips in the proximal intestine. The results of this study suggest that these yeasts can replace up to 40% of fishmeal under current inclusion levels in diets for rainbow trout without compromising growth performance, nutrient digestibility or intestinal health.

Introduction

Alternative protein sources to fish meal in aquaculture diets should be of comparable nutritional value, without compromising the intestinal health of fish. In order to facilitate sustainable production of aquafeeds, these alternatives should not compete with human food sources or utilise arable land for production. Plant protein sources, such as soy and other legumes, are still the main alternative to fish meal in most commercial fish diets, despite issues relating to anti-nutritional compounds (Gatlin *et al.*, 2007). However, the long-term use of plant protein sources as a high-quality protein for the rapidly expanding aquaculture industry is questionable. The availability of plant protein sources for animal feed may become limited over time due to human population growth and lack of viable agricultural land for major production increases (Brown, 2012).

Single-cell protein (SCP), such as yeast, bacteria and microalgae, may be a sustainable alternative to fish meal in aquafeeds but are not currently included as major protein sources in commercial fish diets, except as probiotics or as other additives at low inclusion levels (i.e. <5% dietary inclusion) (Martínez Cruz *et al.*, 2012, Navarrete and Tovar-Ramirez, 2014). Such SCP can be produced using industrial by-products as substrates, allowing long-term sustainable use of resources (Nasseri *et al.*, 2011). A considerable amount of research has been performed on the effects of feeding yeasts to salmonids (Mahnken *et al.*, 1980, Rumsey *et al.*, 1990, Rumsey *et al.*, 1991, Li and Gatlin Iii, 2003, Abdel-Tawwab *et al.*, 2008, Refstie *et al.*, 2010, Øverland *et al.*, 2013, Abro *et al.*, 2014, Hauptman *et al.*, 2014, Vidakovic *et al.*, 2015). However, no studies have focused on high inclusion levels (i.e. >20% dietary

inclusion) of intact baker's yeast in diets for rainbow trout (*Oncorhynchus mykiss*). Whole dried baker's yeast (*Saccharomyces cerevisiae*) has a reported protein content of above 450 g kg^{-1} dry matter (DM) and an amino acid profile characterised by slight methionine deficiency (Vidakovic *et al.*, 2015, Langeland *et al.*, 2016). In addition, Vidakovic *et al.* (2015) observed lower methionine digestibility in diets containing intact *S. cerevisiae* than in a fish meal-based reference diet. Several other authors have indicated potential for using crystalline methionine supplementation in diets with *S. cerevisiae* to achieve minimum nutrient requirements and maintain adequate growth (Murray and Marchant, 1986, Gaylord *et al.*, 2010, Hauptman *et al.*, 2014).

The yeast *Wickerhamomyces anomalus*, formerly known as *Hansenula* and *Pichia anomala*, is a species characterised by efficient utilisation of various organic substrates, similar protein content to *S. cerevisiae* and high phytase activity (Vohra and Satyanarayana, 2001, Olstorpe *et al.*, 2009). High phytase activity of *W. anomalus* may confer an additional advantage of breaking down phytic acid in diets and consequently increasing phosphorus retention in fish, while reducing phosphorus discharge. Existing literature suggests that phytase is able to remain active at 80° C for up to 15 minutes and is likely to be de-activated during extrusion processing at temperatures above 100 ° C (Vohra and Satyanarayana, 2001, Kumar *et al.*, 2012). However, study by Huyben *et al.* (2017a) demonstrated that yeast is able to survive the extrusion temperatures and could potentially produce phytase during feed storage and digestion.

Same authors demonstrated that feeding *W. anomalus* resulted in similar amino acid uptake in rainbow trout compared with fish meal, but the effects of this yeast on growth performance and digestibility in fish are unknown.

The main aim of this study was to investigate the effects of feeding diets with graded replacement of fish meal with the yeast *S. cerevisiae* or a mixture of *W. anomalus* and *S. cerevisiae* on growth performance, nutrient digestibility, nutrient retention and intestinal morphology in rainbow trout. The need for crystalline methionine supplementation in diets with yeast was also studied.

Materials and Methods

Facilities and fish

The experiment was carried out at Kälarne Research Station (Vattenbrukscentrum Norr AB, Kälarne, Sweden) and the experimental period was 10 weeks (July to October 2014). Four weeks before the experiment, 840 rainbow trout weighing 93.7 ± 3.8 g (mean ± S.D.) were netted and anesthetised with 100 mg L^{-1} tricaine methane sulphonate (MS-222 Western Chemical Inc., Ferdale, WA, USA). Fish were randomly allocated (35 fish per tank) to 24 cubic fibreglass tanks, each 340 L in volume. The tanks were supplied with 10 L min^{-1} flow-through water with a mean temperature of 12.9 ± 1.2°C that was derived from Lake Ansjön, after passage through a rotating drum filter. Two days before the beginning of the experiment (week 0), fish were netted, anesthetised with 100 mg L^{-1} MS-222 and weighed. This

procedure was repeated at 3, 7 and 10 (end) weeks. Duration of light exposure was set at 12 h during the entire experiment and water temperature was recorded daily. In order to decrease the stocking density and prevent negative fish interactions, 5 fish in week 3 and 15 fish in week 7 were removed from each tank and euthanised with an overdose of MS-222 (300 mg L^{-1}), followed by exsanguination by cutting through the gill arches. The experiment was carried out in compliance with laws and regulations concerning experiments with live animals overseen by the Swedish Board of Agriculture and approved by the Ethics Committee for Animal Experiments in Umeå, Sweden.

Diets and feeding

Before the experiment, fish were fed a commercial diet (3mm Nutra, Skretting AS, Norway) for three weeks and switched to experimental diets for one week to check for diet acceptance. The diets used in the experiment comprised one fish meal-based reference (control) and seven test diets (Table 1). The fish meal (FM) diet was formulated similarly to a commercial diet for rainbow trout, with high-quality, low-temperature dried fish meal as the main protein source. The test diets were based on the FM diet, with replacement of the fish meal with yeast ingredients on a digestible protein basis, according to digestibility values for arctic charr, established in an earlier study by Langeland *et al.* (2016). The yeast *S. cerevisiae* replaced 20% (diet S20), 40% (diet S40) and 60% (diet S60) of fish meal and a 70:30 biomass ratio of the yeasts *W. anomalus* and *S. cerevisiae* replaced 20% (diet W20), 40% (diet W40) and 60% (diet W60) of fish meal. All diets were formulated on a iso-nitrogenous basis, accounting for 10% lower crude protein (CP) digestibility of yeast than fish meal (Vidakovic

126 *et al.*, 2015, Langeland *et al.*, 2016) and based on the nutrient requirements for rainbow trout
127 recommended by NRC (2011). All diets with the exception of diet S60-Met were
128 supplemented with crystalline L-methionine up to a total methionine content of 9 g kg^{-1} diet,
129 i.e. well above the required level of 7 g kg^{-1} diet based on NRC (2011).

130 Molasses was used as a substrate for production of *W. anomalus*, while harvesting was
131 performed using technology developed by Jästbolaget AB (Sweden), which was originally
132 designed for *S. cerevisiae*. In this set-up, it was not possible to obtain a pure fraction of
133 *W. anomalus* with a protein content of nearly 60% previously obtained in laboratory
134 conditions (unpublished data). Therefore, a mixture of *W. anomalus* and *S. cerevisiae* (70:30
135 ratio) was used to obtain a moderate protein content. The chemical composition of the yeasts
136 and diets is given in Tables 2 and 3.

137 The diets were produced by extrusion at the Natural Resources Institute Finland (Laukaa
138 Research Station) with a twin-screw extruder (3 mm die, BC-45 model, Clextral, Creusot
139 Loir, France). All ingredients were mixed in a vertical Metos mixer with the addition of
140 boiling water to a final moisture content of about 20%. During the extrusion process, feed
141 mash was heated to 120-130°C for 30 s, air-dried overnight in a vertical oven at 60°C and
142 then coated with lipids using a vacuum coater (Pegasus PG-10VC, Dinnissen, Sevenum,
143 Netherlands).

144 The diets were distributed daily by automatic feeders (Arvo-Tec T 2000, Huutokoski,
145 Finland) every 20-30 min for 12 h. Feed waste was collected according to Helland *et al.*
146 (1996) using automatic feed waste collectors (Hølland Teknologi, Sandnes, Norway). Each

147 diet was fed to three randomised tanks at near-satiation fixed rations of 1.5% of total fish
148 biomass in each tank. The satiation levels were determined using control (FM) feed in the
149 week prior to the experiment. The fixed feeding rations were selected in order to target the
150 physiological function of the diets and avoid possible compensatory feeding due to
151 nutritional differences. The feed allowance was corrected after each weighing and feeding
152 resumed on the second day after each weighing. Due to incomplete oil absorption during
153 vacuum coating, diets S60, S60-Met, W40 and W60 were found to gradually obstruct the
154 feeders, consequently reducing the feed distribution to the fish. Therefore, feeders
155 distributing diets S60, S60-Met, W40 and W60 were replaced with daily loaded belt feeders
156 (Hølland Teknologi, Sandnes, Norway) for the last period of the experiment (week 7 to 10)
157 thereafter distributing the fixed rations of 1.5% of total fish biomass in each tank. Dry matter
158 (DM) determination of feed and feed waste is described below. Feed intake was calculated
159 as: Feed given DM (g) – (Feed waste DM (g)/recovery), where recovery was determined by
160 the percentage of DM recovered from each diet that passed through empty tanks under the
161 same experimental conditions, according to Helland *et al.* (1996).

162 *Sampling of fish and faeces*

163 Before the start of the experiment, 10 fish from the holding tanks were sampled, euthanised
164 as described above and then stored at -25°C until whole-body analysis was performed. In
165 weeks 3 and 7, 5 and then 15 fish from each tank were removed and euthanised as described
166 previously, and the faeces were collected for analysis of digestibility. At the end of the
167 experiment (week 10), the remaining 15 fish in the experimental tanks were netted and
168 euthanised. Body weight was recorded for each fish. Three fish from each tank were

randomly sampled and used for microbiota sampling in a parallel study by Huyben *et al.* (2017a). Faeces were collected from remaining 12 fish. During this procedure, the distal intestine located after the ileorectal valve was dissected and faeces were collected by gentle scraping with a scalpel without washing. Collected faeces were pooled as one sample per tank for digestibility analysis. Prior to the faeces collection, whole viscera and liver from five fish per tank were removed and weighed to calculate viscerosomatic index (VSI) and hepatosomatic index (HSI). Following the faeces collection, five fish per tank were selected for whole body analysis and the remaining two fish were discarded.

Sample preparation and chemical analysis

Whole fish stored at -25°C were thawed and homogenised with a mixer (B-400, Büchi Labortechnik AG, Flawil, Switzerland). Homogenised fish, experimental feed and faeces were freeze-dried, ground with a coffee grinder (KG40, DeLonghi Appliances, Italy) and stored at -25°C until analysis.

The DM and ash content were determined according to Jennische and Larsson (1990). In brief, the DM content was determined by measuring the weight difference before and after heating the samples in an oven at 103°C for 16 h. Ash content was determined after incineration at 550°C for 3 hours. Total nitrogen (N) was determined using the Kjeldahl method with a digester and analyser (2020 and 2400 Kjeltec, FOSS Analytical A/S, Hilleröd, Denmark) and CP was calculated as N x 6.25 (Nordic Committee on Food Analysis, 1976).

Crude lipid (CL) content was analysed using an extraction system (Soxtec System HT 1043 Extraction Unit, FOSS Analytical A/S, Hilleröd, Denmark) without acid hydrolysis

according to the manufacturer's recommendations (ANKOM Technology, Macedon, NY, USA) with modifications by Hooft *et al.* (2011).

Determination of gross energy (GE) was performed in an isoperibol calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA) and expressed as MJ kg^{-1}. Inert marker, TiO_2, was analysed according to Short *et al.* (1996). Nutrient detergent fibre content (NDF) was analysed by the amylase neutral detergent method according to Mertens (2002).

The amino acid (AA) content of diets and faeces was analysed at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden) by ion exchange high-performance liquid chromatography, according to ISO-13903 (2005). In brief, samples were oxidised for 16 h with performic acid and then hydrolysed for 23 h with 6M HCl. Individual AA were separated on an ion-exchange chromatograph (Biochrom 30 amino acid analyser, Biochrom Ltd., Cambridge, England) and the peaks were identified, integrated and quantified with EZChrom Elite (Biochrom Ltd., Cambridge, England).

Determination of phosphorus content was performed by plasma emission spectroscopy (Spectro Analytical Instruments GmbH & Co., Kleve, Germany) at a certified laboratory (Agrilab AB, Uppsala, Sweden) after extraction of samples with HNO_3 as described by Bahlsberg-Pålsson (1990).

Intestinal morphology

Proximal and distal intestinal tissues were collected from four fish per tank using the ileorectal valve as the indicator of the transition from proximal into distal intestine, fixed in phosphate-buffered (0.1 mM, pH 7.2) 4% formalin for 24 h at 4°C, washed in 0.9% NaCl and stored in 70% ethyl alcohol (EtOH) until histology was performed. Tissues were dehydrated through an alcohol gradient and Histolab-clear (Histolab Products AB, Gothenburg, Sweden) and embedded in paraffin wax using standard procedures. Sections (7 μm) of the proximal intestine were produced with a Shandon finesse microtome (Thermo Fisher Scientific, Waltham, MA, USA), mounted on 3′-aminopropyltriethoxysilane (APES; Sigma-Aldrich)-coated slides and dried at 37°C for 24 h. Tissue slides were stained with a combination of haematoxylin-eosin and alcian blue 8 GX, pH 2.5. The sections were examined under a Nikon eclipse E1000 microscope and photographs taken with a Nikon DXM1200 camera (Nikon Instruments Europe, Amsterdam, Netherlands). Intestinal sections (n=8-12) from the proximal intestine were analysed for three different morphological parameters. From each fish, four non-overlapping areas per intestine were assessed for mucosal fold height and width (μm) and goblet cells per mm epithelium, using Biopix imaging software (Biopix AB, Gothenburg, Sweden). Histological samples were randomised and blindly evaluated.

Calculations

Weight gain (WG), specific growth rate (SGR) and feed conversation ratio (FCR) were calculated using the following equations:

231 WG (%) = ((FW – SW)/SW) ×100

232 SGR (% day^{-1}) = 100× ((*ln* FW – *ln* SW)/T)

233 FCR = FI / (FW-SW)

234 RFI (% of body weight day^{-1})= 100x (FI / (T x (SW + FW)/2))

235 where RFI is the relative feed intake over the whole experimental period expressed in
236 percentage of body weight, FW is the final weight (g) of the fish, SW is the initial weight of
237 the fish (g), T is the duration of the experiment (days) and FI is total feed intake (g) on an
238 DM basis.

239 Hepatosomatic index and viscerosomatic index were calculated according to the following
240 equations:

241 HSI (%) = $(W_{Liv}/FW) \times 100$

242 VSI (%) = $(W_{Vis}/FW) \times 100$

243 where W_{Liv} is the weight of liver (g), W_{Vis} is the weight of viscera (g) and FW is fish weight.
244 The VSI values include the faeces weight (≤ 0.5 g).

245 Nutrient retention was determined as:

246 (Nutrient retained in the body/Nutrient ingested) × 100.

247 Apparent digestibility coefficients (ADC) were calculated as:

248 ADC_{diet} (%) = $[1 – (F/D \times D_i/F_i)]\times100$

249 ADC DM (%) = $[1 – (D_i/F_i)] \times 100$

250 where F is % nutrient (or kJ g^{-1}GE) in faeces, D is % nutrient (or kJ g^{-1}GE) in diet, D_i is %
251 inert marker in diet and F_i is % inert marker in faeces.

252 *Statistical analysis*

253 The effects of diet on growth performance (FW, WG and SGR), FCR, ADC, relative feed
254 intake (RFI), protein and gross energy retention and relative organ weight (HSI and VSI)
255 were evaluated using the model PROC MIXED, including the fixed factor of test diet and the
256 random factor of tank. Significant effects of the diets were determined using *post hoc* least
257 squared means (LSMEANS) with Tukey's adjustment for multiple pair-wise comparisons.
258 Tank was the experimental unit and significance level was set to $p<0.05$. All data were
259 normally distributed and analyses were performed using Statistical Analysis System version
260 9.3 (SAS Institute Inc., NC, USA).

261

262 **Results**

263 *Growth performance, body indices, nutrient retention and body composition*

264 In terms of growth performance, no differences were observed for FW and WG between
265 different treatments (Table 4). However, tendency ($p=0.06$, Table 4) for lower final weight
266 was observed for fish fed diets S60 and W60. For fish fed diet W60 significantly lower SGR
267 was observed, while fish fed diets S60 and S60-Met SGR did not differ from fish fed the FM
268 diet. No significant differences ($p=0.64$) in FCR were observed among any of the diets.

Assessments of relative body indices showed that fish fed diet W20 had significantly higher HSI than fish fed diet W40. There were no differences in VSI between fish fed different diets.

There were no differences in nutrient retention (CP, CL, energy and phosphorus) between fish fed the different diets (Table 4). Phosphorus retention tended to differ (P=0.06) among the different dietary groups. Highest retention was observed for diet S60 and lowest for diet W20.

The whole body composition did not differ between different diets, with the exception of CL content which was significantly lower in fish fed S60 diet than in fish fed FM, S20 and S40 diets.

Relative feed intake and apparent digestibility

The relative feed intake (RFI) varied from 0.91 to 1.18 % of BW day^{-1} for total duration of the experiment (Table 4). The RFI in fish fed diet S40 was higher than in fish fed diet S60. The average feed waste reported per tank (± SEM) was 285.7 ± 74.8 g during the whole experimental period.

The values for apparent digestibility of DM were higher in fish fed FM diet than in fish fed diet S60-Met. Apparent digestibility of CP was higher for fish fed the FM diet than fish fed the yeast-based diets, except for diet W20 (Table 5). In addition, significantly higher ADC of CP was found for fish fed diet W20 than fish fed diets S60 and S60-Met. The highest ADC for sum of indispensable amino acids (IAA) was recorded for fish fed the FM diet, which again did not vary from fish fed diet W20. For fish fed diet W20, ADC of sum of IAA was significantly higher than for fish fed diets S60, S60-Met and W60. Similarly, ADC of all

individual IAA, except threonine, was typically highest for fish fed the FM and W20 diets, while the lowest ADC was found for fish fed diets S60 and S60-Met. The highest ADC of threonine was recorded in fish fed the FM diet. Lastly, ADC of phosphorus was significantly higher for fish fed diet W20 than for fish fed the FM diet.

Faeces collected from fish fed different diets varied greatly in respect to DM content. The lowest DM (14.9%) was found in fish fed diet W60 and the highest (17.3%) in fish fed the FM diet.

Intestinal morphology

In the proximal region of the intestine, there were no apparent effects of diet on the height of the villi (Table 4). All dietary treatments, including FM, displayed oedematous mucosal fold tips. The mean width of the oedema was significantly enhanced in fish fed diet S60 compared with fish fed the FM diet (Figure 1). However, the more pronounced oedema observed in fish fed diet S60 was not infiltrated by any immune cells and therefore was not classified as enteritis. Number of goblet cells was highest in fish fed the FM diet, but no significant differences were observed between the different diets (Table 4).

Discussion

In terms of growth performance, earlier studies such as that of Hauptman *et al.* (2014) reported that replacing more than 37.5% (11.2% dietary inclusion) of fish meal with grain distiller's dried yeast in diets for rainbow trout resulted in decreased growth performance. In earlier work (Vidakovic *et al.* (2015), we found that 40% fish meal replacement (28.9%

311 dietary inclusion) with *S. cerevisiae* resulted in lowered growth performance of Arctic charr
312 (*Salvelinus alpinus*). However, fish meal replacement with yeast in the present experiment
313 was based on digestible protein rather than crude protein, which may have indirectly
314 improved fish performance to match that of the FM diet (Table 3). On the other hand, the
315 results of the present study are in line with findings by Langeland *et al.* (2016), who in a
316 series of digestibility experiments observed similar growth rates of Arctic charr fed 30%
317 dietary inclusion of *S. cerevisiae* and an FM-based reference diet. However, Langeland *et al.*
318 (2016) fed the fish *ad libitum*, which resulted in higher dietary feed intake in fish fed diets
319 with yeast compared with in the present study, where fixed rations were applied.

320 Previous research by de la Higuera *et al.* (1981) has shown that when diets for rainbow trout
321 contain *W. anomalus* as the only protein source (812 g kg^{-1} diet), feed intake and growth
322 decreased significantly. The present study showed that when fish meal was replaced with
323 *S. cerevisiae* as well as *W. anomalus* and *S. cerevisiae* mix at 60 % or at 32.1 and 35.5 %
324 dietary inclusion, there were negative effects on feed intake. However, at the same time the
325 FCR, FW and WG were not significantly affected. While the decreased feed intake of diet
326 S60 may be a function of the observed decreased daily feed ration (DFR) caused by feed
327 delivery issues, the decreased intake of diet W60 could be a result of lower preference for
328 this diet. In addition, fish fed diet W60 had lower SGR than fish fed FM and S20 diet. This
329 may as well be a combined effect of lower feed intake and poorer protein quality. Despite
330 the comparable CP levels between the diets, the actual AA content per unit CP was lower in
331 the *W. anomalus* and *S. cerevisiae* mix (Table 2), indicating a possibly lower biological value
332 of this protein source due to higher non-protein nitrogen content. It remains uncertain

however whether prolonged feeding with test diets would amplify the slight differences in growth. Additionally, it is possible that if *ad libitum* feeding had been applied in the present study, differences in growth performance would have been amplified.

A possible explanation for slightly reduced DFR for diets S60, S60-Met and W 40 could be the observed poorer physical feed pellet quality and oil absorption, causing decreased delivery of feed by feeders during trials and thus lower feed intake by the fish, which was especially evident for diet S60. In addition, the feed recovery test (Table 5) shows that the pellets containing higher levels of *S.cerevisiae* + *W. anomalus* mix had lower recovery rate when compared with most diets, indicating that the pellets dissolved quicker in water. On the contrary, the recovery of S20 diet was improved when compared to FM diet, illustrating possible beneficial effect on pellet quality when *S. cerevisiae* is added at lower inclusion rates. Similar observations have been reported previously for diets with high inclusion of yeast extract (Langeland *et al.*, 2016). Hauptman *et al.* (2014) also observed alterations in physical pellet quality in diets with yeast and indicated strong correlation between yeast inclusion rate and the pellet loss during Holmen durability pellet testing. Aas *et al.* (2011) found that the physical pellet quality could modify the rate of passage in rainbow trout and consequently affect the nutrient utilization in trout. Poor oil absorption was also observed for diets W40 and W60 while the fish fed diet W60 achieved lowest growth performance when measured as SGR. However, observations regarding physical pellet quality and oil absorption could not be confirmed in the present study, as no physical pellet quality analysis was performed. Recent studies have suggested modifying conditions during extrusion as a means to improve the digestibility of yeast protein (Vidakovic *et al.*, 2015, Langeland *et al.*, 2016).

356 Other authors have emphasised the importance of feed processing aspects when working with
357 extruded diets (Aguilar-Uscanga and François, 2003, Klis *et al.*, 2006, Baeverfjord *et al.*,
358 2006). More research is needed in order to optimise the production process to improve pellet
359 quality and nutrient delivery of diets with high inclusion of yeast.

360 The lack of difference in growth performance of fish fed methionine-enriched and non-
361 enriched diets could have several explanations. The total methionine content of diet S60-Met
362 (7.8 g kg^{-1} DM) was slightly above the minimum requirement for rainbow trout (7 g kg^{-1} DM
363 (NRC, 2011), even with no addition of crystalline methionine. However, the requirements
364 were established on the assumption that bioavailability of these amino acids is close to 100%,
365 which is rarely the case in practical diets. Moreover, the sum of methionine and cysteine for
366 diet S60-Met was 13.9 g kg^{-1} DM, which is well above the requirement of 11 g kg^{-1} DM set
367 by NRC (2011), and the digestibility of methionine was therefore still sufficient to meet this
368 requirement. Huyben *et al.* (2017b) fed dorsal aorta-cannulated rainbow trout the same basic
369 diets as in this study (FM, 60S and 60W) and found that post-prandial plasma levels of
370 methionine were significantly higher in fish fed the yeast diets compared with fish fed the
371 FM diet. Those authors suggested that the higher methionine supplementation in the yeast-
372 based diets created a surplus of free methionine in the plasma and proposed that dietary
373 supplementation may not be necessary.
374
375 Studies by Vidakovic *et al.* (2015) and Langeland *et al.* (2016) report lower apparent
376 digestibility of protein in diets for Arctic charr containing *S. cerevisiae* than in diets
377 containing fish meal. Apparent digestibility of all IAA in the present study, with the

exception of threonine, was highest for fish fed the FM and W20 diets. Moreover, the ADC of IAA for all experimental diets were either above or slightly below 90 % which is higher than what was reported in earlier studies using yeast protein sources for salmonids.

The reduced ADC of IAA in diets S60, S60-Met and W60 may indicate lower limiting inclusion levels of these ingredients. Additionally, the total amount of IAA per unit crude protein differed between the two yeast products used (Table 2), possibly explaining slightly lower growth in fish fed diets containing a mix of *W. anomalus* and *S. cerevisiae* compared with fish fed diets containing *S. cerevisiae*.

The higher phosphorus ADC in fish fed diet W20 compared with fish fed diet FM may indicate a possible effect of phytase activity by the yeast *W. anomalus.* In fact, fish fed all experimental diets had numerically higher phosphorus digestibility than fish fed the FM diet. Huyben *et al.* (2017a) used the same diets as in the current trial and found that there was a reduction in number and abundance of culturable yeast cells in the diets after the extrusion, however yeast containing diets still had relatively high abundance of culturable yeast cells. These surviving yeast may still be a source of phytase in the diets after the extrusion. Additionally, it has been suggested that diets can have lower digestibility of phosphorus in the presence of fishmeal due to the higher calcium content (NRC, 2011). Lowest retention of phosphorus was observed in fish fed diet W20, while having the highest phosphorus ADC at the same time. The reason for this is currently unknown but such results can point to a possible analytical error. To the best of our knowledge, there are no published studies on the phytase activity of yeasts in salmonids. Our observation therefore indicates a possible direction for future work, especially when using yeast in fish diets to improve phosphorus retention and consequently reduce phosphorus emissions to the environment.

The more pronounced oedema in the mucosal fold of the proximal intestine in the fish fed S60 diet indicates reduced intestinal health (Figure 1). This was most likely a result of the diet, and is in agreement with reduced growth in fish fed the S60 diet. In mammals, oedema can be a result of stress-induced reduction of the barrier function of the microvasculature induced by mast cell activation and can result in fluid leakage and accumulation in the villi (Wilson and Baldwin, 1999). Furthermore, oedematous villi can be associated with general inflammation (Serra and Jani, 2006). Although the oedema observed in this study was not apparently infiltrated by immune cells, the possibility of an early stage of inflammation, more severe in fish fed S60 diet, cannot be excluded. In addition, oedema can be related to hypoxic conditions in the enterocytes. The tip of the villi are normally hypoxic, but the hypoxic area can extend further down in the villi during neutrophil infiltration and/or decreased blood perfusion of the intestine (Colgan and Taylor, 2010). Huyben *et al.* (2016) who fed rainbow trout the same diets as in the present study found that fish fed the yeast-based diets displayed signs of haemolytic anaemia. Those authors suggested that high levels of nucleic acids in yeast-based diets could overwhelm anti-oxidative processes and impair red blood cells, consequently leading to cell lysis, and recommended limited use of yeasts in fish diets. It can therefore be proposed that haemolytic anaemia was one reason for the possible hypoxia-induced oedema observed in the present study. Further studies are needed to confirm the aetiology behind intestinal oedema in rainbow trout.

Except for fish fed diet S20, faeces DM gradually decreased with increased yeast inclusion level (Table 3), which indicates that the yeast induced signs of diarrhoea. This is also

supported by a decreased ADC of DM in fish fed diet S60-Met, compared to fish fed FM diet. In yeast cells, 10-25% of cell biomass may be represented by cell walls and these contain high proportions of chitin (Klis *et al.*, 2006), which has been shown to induce diarrhoea in fish when given in high amounts (Lindsay *et al.*, 1984, Shiau and Chin, 1999, Olsen *et al.*, 2006, Kraugerud *et al.*, 2007). However, the NDF content of the 60% yeast-based diets was less than half that of the fish meal diet (Table 3), possibly due to lower cellulose inclusion (Table 1), which may have affected intestinal mucus secretion. Threonine is an IAA present in high concentrations in mucins (NRC, 2011) and fish are known to produce excessive mucus in stressful conditions (Eddy and Fraser, 1982, Khan and McGeer, 2013). Previous studies by Vidakovic *et al.* (2015) showed that feeding Arctic charr a diet with 28.9% dietary inclusion of intact *S. cerevisiae* resulted in disruption of the intestinal barrier function and coincided with decreased ADC of threonine. In the present study, low ADC values for threonine, coupled with lower faecal DM for fish fed all experimental diets except FM, indicate increased intestinal mucus excretion. In view of these results, together with observations on intestinal morphology, presence of intestinal stress in fish fed diets with higher yeast inclusion levels cannot be ruled out. Therefore, the impact of yeast and the role of threonine ADC as an indicator of increased mucus production in the intestines merit further examination.

Based on the feed formulation used in this study, it can be concluded that methionine supplementation of diets with high *S. cerevisiae* inclusion is not required. Furthermore, findings of intestinal inflammation in fish fed diet S60 indicate that such a high inclusion rate cannot be recommended, as it may have negative effects on fish. Hence, further research

focusing on possible anti-nutritional effects of yeasts is needed in order to develop these SCPs in diets for salmonids. It can be concluded that both *S. cerevisiae* and a 70:30 mix of *W. anomalus* can replace up to 40% of fish meal protein without negative effects on growth performance, nutrient retention or intestinal health. To the best of our knowledge, such high inclusion rates of yeasts in fish diets without reductions in growth and health have not been achieved previously. Observations related to poor lipid absorption in high yeast inclusion diets point to a need for further studying the effects of yeast inclusion on physical pellet quality.

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References

Aas, T.S., Terjesen, B.F., Sigholt, T., Hillestad, M., Holm, J., Refstie, S., Baevefjord, G., Rørvik, K.-A., Sørensen, M., Oehme, M. & Åsgård, T. (2011) Nutritional responses in rainbow trout (Oncorhynchus mykiss) fed diets with different physical qualities at stable or variable environmental conditions. *Aquaculture Nutrition,* **17,** 657-670.

Abdel-Tawwab, M., Abdel-Rahman, A.M. & Ismael, N.E.M. (2008) Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with *Aeromonas hydrophila*. *Aquaculture,* **280,** 185-189.

Abro, R., Moazzami, A., Lindberg, J. & Lundh, T. (2014) Metabolic insights in Arctic charr (*Salvelinus alpinus*) fed with zygomycetes and fish meal diets as assessed in liver using nuclear magnetic resonance (NMR) spectroscopy. *International Aquatic Research,* **6,** 1-11.

Aguilar-Uscanga, B. & François, J.M. (2003) A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Letters in Applied Microbiology,* **37,** 268-274.

Baeverfjord, G., Refstie, S., Krogedal, P. & Åsgård, T. (2006) Low feed pellet water stability and fluctuating water salinity cause separation and accumulation of dietary oil in the stomach of rainbow trout (Oncorhynchus mykiss). *Aquaculture,* **261,** 1335-1345.

Bahlsberg-Pålsson, A.M. (1990) *Förbehandling, uppslutning och extraktberedning av växt-och förnaprov,* Lunds Universitet, Lund, Sweden.

Brown, L.R. (2012) *Full Planet, Empty Plates: The New Geopolitics of Food Scarcity,* W. W. Norton.

Colgan, S.P. & Taylor, C.T. (2010) Hypoxia: an alarm signal during intestinal inflammation. *Nature reviews. Gastroenterology & hepatology,* **7,** 281-287.

de la Higuera, M., Sánchez-Muniz, F.J., Mataix, F.J. & Varela, G. (1981) Nitrogen utilization by rainbow trout (*Salmo gairdneri*) fed on the yeast *Hansenula anomala*. *Comparative Biochemistry and Physiology Part A: Physiology,* **69,** 583-586.

Eddy, F.B. & Fraser, J.E. (1982) Sialic acid and mucus production in rainbow trout (*Salmo gairdneri* Richardson) in response to zinc and seawater. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* **73,** 357-359.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R. & Wurtele, E. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research,* **38,** 551-579.

Gaylord, T.G., Barrows, F.T. & Rawles, S.D. (2010) Apparent amino acid availability from feedstuffs in extruded diets for rainbow trout *Oncorhynchus mykiss*. *Aquaculture Nutrition,* **16,** 400-406.

Hauptman, B.S., Barrows, F.T., Block, S.S., Gibson Gaylord, T., Paterson, J.A., Rawles, S.D. & Sealey, W.M. (2014) Evaluation of grain distillers dried yeast as a fish meal substitute in practical-type diets of juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* **432,** 7-14.

Helland, S.J., Grisdale-Helland, B. & Nerland, S. (1996) A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture,* **139,** 157-163.

Hooft, J.M., Elmor, A.E.H.I., Encarnação, P. & Bureau, D.P. (2011) Rainbow trout (*Oncorhynchus mykiss*) is extremely sensitive to the feed-borne Fusarium mycotoxin deoxynivalenol (DON). *Aquaculture,* **311,** 224-232.

Huyben, D., Nyman, A., Vidaković, A., Passoth, V., Moccia, R., Kiessling, A., Dicksved, J. & Lundh, T. (2017a) Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. *Aquaculture,* **473,** 528-537.

Huyben, D., Vidaković, A., Langeland, M., Nyman, A., Lundh, T. & Kiessling, A. (2017b) Effects of dietary yeast inclusion and acute stress on postprandial plasma free amino acid profiles of dorsal aorta-cannulated rainbow trout. *Aquaculture Nutrition,* **43,** 421-434.

Huyben, D., Vidakovic, A., Nyman, A., Langeland, M., Lundh, T. & Kiessling, A. (2016) Effects of dietary yeast inclusion and acute stress on post-prandial whole blood profiles of dorsal aorta-cannulated rainbow trout. *Fish Physiology and Biochemistry***,** 1-14.

ISO-13903 (2005) ISO-13903 In *Animal feeding stuffs, determination of amino acid content.* . International Organization for Standardization, Geneve, Switzerland.

Jennische, P. & Larsson, K. (1990) Traditionella svenska analysmetoder för foder och växtmaterial, Rapport 60. (Laboratorium, S.L. ed. Statens Lantbrukskemiska Laboratorium, Uppsala, Sweden.

Khan, F.R. & McGeer, J.C. (2013) Zn-stimulated mucus secretion in the rainbow trout (*Oncorhynchus mykiss*) intestine inhibits Cd accumulation and Cd-induced lipid peroxidation. *Aquatic Toxicology,* **142–143,** 17-25.

Klis, F.M., Boorsma, A. & De Groot, P.W.J. (2006) Cell wall construction in *Saccharomyces cerevisiae*. *Yeast,* **23,** 185-202.

Kraugerud, O.F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, Å. & Svihus, B. (2007) Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. *Aquaculture,* **273,** 96-107.

Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G. & Becker, K. (2012) Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition,* **96,** 335-364.

Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J.E., Kiessling, A. & Lundh, T. (2016) Digestibility of microbial and mussel meal for Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*). *Aquaculture Nutrition,* **22,** 485-495.

Li, P. & Gatlin Iii, D.M. (2003) Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops*×*M. saxatilis*). *Aquaculture,* **219,** 681-692.

Lindsay, G.J.H., Walton, M.J., Adron, J.W., Fletcher, T.C., Cho, C.Y. & Cowey, C.B. (1984) The growth of rainbow trout *(Salmo gairdneri*) given diets containing chitin and its relationship to chitinolytic enzymes and chitin digestibility. *Aquaculture,* **37,** 315-334.

Mahnken, C.V.W., Spinelli, J. & Waknitz, F.W. (1980) Evaluation of an alkane yeast (*Candida sp*.) as a substitute for fish meal in Oregon Moist Pellet: Feeding trials with coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*). *Aquaculture,* **20,** 41-56.

Martínez Cruz, P., Ibáñez, A.L., Monroy Hermosillo, O.A. & Ramírez Saad, H.C. (2012) Use of Probiotics in Aquaculture. *ISRN Microbiology,* **2012,** 916845.

Mertens, D.R. (2002) Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *J AOAC Int,* **85,** 1217-1240.

Murray, A.P. & Marchant, R. (1986) Nitrogen utilization in rainbow trout fingerlings (*Salmo gairdneri* Richardson) fed mixed microbial biomass. *Aquaculture,* **54,** 263-275.

Nasseri, A.T., Rasoul-Amini, S., Morowvat, M.H. & Ghasemi, Y. (2011) Single Cell Protein: Production and Process. *American Journal of Food Technology,* **6,** 103-116.

Navarrete, P. & Tovar-Ramirez, D. (2014) *Use of Yeasts as Probiotics in Fish Aquaculture, Sustainable Aquaculture Techniques,* InTech.

Nordic Committee on Food Analysis (1976) Determination in feeds and faeces according to Kjeldahl, No6. NKML, Oslo, Norway.

NRC (2011) *Nutrient Requirements of Fish and Shrimp,* The National Academies Press, Washington, DC.

Olsen, R.E., Suontama, J., Langmyhr, E., Mundheim, H., RingØ, E., Melle, W., Malde, M.K. & Hemre, G.I. (2006) The replacement of fish meal with Antarctic krill, *Euphausia superba* in diets for Atlantic salmon, *Salmo salar*. *Aquaculture Nutrition,* **12,** 280-290.

Olstorpe, M., Schnürer, J. & Passoth, V. (2009) Screening of yeast strains for phytase activity. *FEMS Yeast Research,* **9,** 478-488.

Øverland, M., Karlsson, A., Mydland, L.T., Romarheim, O.H. & Skrede, A. (2013) Evaluation of *Candida utilis, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*). *Aquaculture,* **402-403,** 1-7.

Refstie, S., Baeverfjord, G., Seim, R.R. & Elvebø, O. (2010) Effects of dietary yeast cell wall [beta]-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (*Salmo salar*) fed sunflower and soybean meal. *Aquaculture,* **305,** 109-116.

Rumsey, G.L., Hughes, S.G. & Kinsella, J.L. (1990) Use of Dietary Yeast *Saccharomyces cerevisiae* Nitrogen by Lake Trout. *Journal of the World Aquaculture Society,* **21,** 205-209.

570 Rumsey, G.L., Hughes, S.G., Smith, R.R., Kinsella, J.E. & Shetty, K.J. (1991) Digestibility and
571 energy values of intact, disrupted and extracts from brewer's dried yeast fed to rainbow trout
572 (*Oncorhynchus mykiss*). *Animal Feed Science and Technology,* **33,** 185-193.

573 Serra, S. & Jani, P.A. (2006) An approach to duodenal biopsies. *Journal of Clinical Pathology,* **59,**
574 1133-1150.

575 Shiau, S.-Y. & Chin, Y.-H. (1999) Estimation of the dietary biotin requirement of juvenile hybrid
576 tilapia, *Oreochromis niloticus*×*O. aureus*. *Aquaculture,* **170,** 71-78.

577 Short, F.J., Gorton, P., Wiseman, J. & Boorman, K.N. (1996) Determination of titanium dioxide
578 added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology,* **59,**
579 215-221.

580 Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling, A. &
581 Lundh, T. (2015) Evaluation of growth performance and intestinal barrier function in Arctic Charr
582 (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel
583 (*Mytilus edulis*). *Aquaculture Nutrition,* **22,** 1348-1360.

584 Vohra, A. & Satyanarayana, T. (2001) Phytase production by the yeast, *Pichia anomala*.
585 *Biotechnology Letters,* **23,** 551-554.

586 Wilson, L.M. & Baldwin, A.L. (1999) Environmental Stress Causes Mast Cell Degranulation,
587 Endothelial and Epithelial Changes, and Edema in the Rat Intestinal Mucosa. *Microcirculation,* **6,**
588 189-198.

589

590

591 Table 1. Formulation of the experimental diets (g kg^{-1}, as-is)

	Diet[1]							
Ingredient	**FM**	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**
Fish meal	300	240	180	120	120	240	180	120
Soy protein concentrate	135	135	135	135	135	135	135	135
Wheat gluten	120	120	120	120	120	120	120	120
Fish oil	110	115	120	125	125	115	120	124
Rapeseed oil	50	50	50	50	50	50	50	50
Wheat meal	60	60	60	60	60	60	60	60
Wheat starch	100	75	45	10	10	65	20	0
Min-vit premix[a]	15	15	15	15	15	15	15	15
Monocalcium phosphate	10	10	10	10	10	10	10	10
β-Cellulose	93	65	42	24	29	64	41	0
Titanium oxide	5	5	5	5	5	5	5	5
L-methionine	2	3	4	5	0	3	5	6
S. cerevisiae	-	107	214	321	321	-	-	-
W. anomalus + *S. cerevisiae*	-	-	-	-	0	118	239	355

592 [1]Fish meal-based reference diet (FM) and diets with 20%, 40% and 60%
593 fish meal protein replaced with *Saccharomyces cerevisiae* (S20, S40, S60)
594 or a 70:30 mix (biomass ratio) of *Wickerhamomyces anomalus* and *S.*
595 *cerevisiae* (W20, W40, W60), with an additional *S. cerevisiae* diet without
596 methionine supplementation (S60-Met).

597 [a]Mineral-vitamin premix contains (per kg): retinol acetate 400000 IU,
598 cholecalciferol 150000 IU, all-race-tocopheryl acetate 15000 IU, menadion
599 sodium bisulfite 500mg, thiamine HCl 1 g, riboflavin 1.5 g, calcium d-
600 pantothenate 4.5 g, biotin 150 mg, folic acid 300 mg, vitamin B12 0.02 mg,
601 niacin 6 g, pyridoxine HCl 1 g, ascorbic acid (Stay C) 15 g, inositol 10 g,
602 zinc 7.5 g, manganese 3 g, iodine 200 mg.

603

604 Table 2. Proximate chemical composition, amino acid profile (g kg^{-1} DM)

605 and energy content (MJ kg^{-1} DM) of intact baker's yeast (*Saccharomyces*

606 *cerevisiae*) and yeast mix (*Wickerhamomyces anomala* and *S. cerevisiae*)

	Ingredient	
	S. cerevisiae	***W. anomalus* + *S. cerevisiae*[3]**
Crude protein	466	422
Sum of amino acids	423.5	360.4
Crude lipid	10	9
Ash	63	70
Gross energy	19.9	20.4
Indispensable amino acids		
Arginine	22.2	18.8
Histidine	9.9	7.9
Isoleucine	23.3	20.5
Leucine	32.6	28.4
Lysine	36.3	30.3
Methionine	7.1	4.9
Phenylalanine	19.5	17.2
Threonine	22.5	19.5
Valine	27.2	22.2
Sum	200.5	169.8
Dispensable amino acids		
Alanine	24.9	21.5
Aspartic acid	45.0	37.6
Cysteine[1,2]	5.8	4.0
Glutamic acid	67.4	58.0
Glycine	21.6	18.0
Ornithine	0.5	0.6
Proline	17.5	15.0
Serine	23.1	21.5
Tyrosine	17.1	14.4
Sum	223.0	190.6

607 [1]Amount present after oxidation of cysteine and cystine to cysteic acid.

608 [2]Conditionally indispensable (NRC, 2011).

609 [3]Mixture of 70:30 *W. anomala* to *S. cerevisiae*

610

611

612 Table 3. Proximate chemical composition, amino acid profile (g kg^{-1} DM) and energy content (MJ

613 kg^{-1} DM) of the experimental diets and faecal dry matter

	Diet[1]							
	FM	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**
Dry matter (%)	92.4	91.1	91.9	91.3	90.4	91.8	92.3	93.3
Crude protein	425	433	440	454	453	432	446	463
Total amino acids	387	389	392	382	416	366	399	393
Crude lipid	196	207	208	203	192	208	200	186
NDF[2]	113.9	88.2	63.9	44.9	44.9	81.7	68.0	25.4
Ash	68.4	66.4	62.8	62.6	59.6	65.2	63.2	61.6
Gross energy	23.6	23.6	23.7	23.9	23.9	23.6	23.6	23.8
Phosphorus	9.3	9.7	9.8	9.8	10.1	10.7	10.1	9.8
Indispensable amino acids								
Arginine	22.1	22.4	22.0	21.3	22.6	20.4	21.9	21.7
Histidine	9.5	9.3	9.5	9.4	9.7	8.8	8.9	9.4
Isoleucine	16.5	16.8	16.8	16.9	18.5	16.1	17.2	17.4
Leucine	30.1	30.3	29.5	28.7	31.9	29.0	30.6	30.2
Lysine	24.1	24.4	24.6	24.4	26.6	22.6	24.4	24.3
Methionine	11.1	12.2	11.4	11.6	7.8	11.3	12.4	12.3
Phenylalanine	18.6	18.9	19.0	18.9	20.8	17.6	19.8	19
Threonine	15.4	15.5	16.3	15.7	16.3	14.3	16.3	16.3
Valine	19.6	19.6	19.9	19.6	21.2	18.2	19.6	20.6
Sum	167.0	169.4	169.0	166.5	175.4	158.3	171.1	171.2
Dispensable amino acids								
Alanine	19.8	19.9	20	19	20.8	19.1	20.0	19.7
Aspartic acid	34.7	34.4	35.1	34.2	37.8	32	34.6	35.1
Cysteine[3,4]	5.7	5.6	6.1	5.8	6.1	5.3	5.5	5.6

Glutamic acid	80.3	80.8	81.4	79	91.1	76.9	86.8	82
Glycine	20	19.5	19	18.1	20.0	18.5	19.3	18.5
Proline	26.2	25.7	26.9	25.8	27.0	24.8	26.5	26.3
Serine	19	18.7	19.5	18.7	21.6	17.5	20.3	19.5
Tyrosine	14.9	15.2	15.7	15.3	16.3	13.8	15.2	15.3
Sum	220.6	219.8	223.7	215.9	240.7	207.9	228.2	222.0
Faeces								
Dry matter (%)	17.3	15.3	16.5	16.8	15.8	16.3	15.4	14.9

[1]Fish meal-based reference diet (FM) and diets with 20%, 40% and 60% fish meal protein replaced with *Saccharomyces cerevisiae* (S20, S40, S60) or a 70:30 mix (biomass ratio) of *Wickerhamomyces anomalus* and *S. cerevisiae* (W20, W40, W60), with an additional *S. cerevisiae* diet without methionine supplementation (S60-Met).

[2]NDF = Neutral detergent fibre.

[3]Amount present after oxidation of cysteine and cystine to cysteic acid.

[4]Conditionally indispensable (NRC, 2011).

623 Table 4. Growth performance, relative organ weight, nutrient retention and intestinal morphology of rainbow trout fed
624 experimental diets. SW = start weight, FW = final body weight, SGR = specific growth rate, WG = weight gain, FCR =
625 feed conversion ratio, DFR = daily feeding rations, RFI = relative feed intake, HSI = hepatosomatic index, VSI =
626 viscerosomatic index. Data presented are least square means ± standard deviation.

	Diet[1]								
	FM	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**	**P-value**
Growth performance[2]									
SW (g)	147.6 ± 1.2	145.3 ± 3.9	146.6 ± 1.2	142.7 ± 4.7	140.2 ± 2.7	142.4 ± 2.9	148.0 ± 1.2	144.6 ± 2.9	0.52
FW (g)	355.0 ± 13.7	345.5 ± 4.3	343.7 ± 21.9	322.5 ± 0.2	315.8 ± 21.0	340.4 ± 17.0	332.0 ± 13.0	288.6 ± 21.0	0.06
SGR (% day^{-1})	1.2^{a} ± 0.0	1.2^{a} ± 0.1	1.2^{ab} ± 0.1	1.2^{ab} ± 0.1	1.2^{ab} ± 0.1	1.2^{a} ± 0.1	1.2^{ab} ± 0.2	1.0^{b} ± 0.1	0.04
WG (%)	139.9 ± 7.4	137.9 ± 15.1	133.9 ± 21.1	126.6 ± 10.9	124.8 ± 9.6	139.3 ± 7.9	124.8 ± 33.6	99.5 ± 4.4	0.06
FCR	0.92 ± 0.1	0.91 ± 0.0	1.0 ± 0.2	0.94 ± 0.1	0.97 ± 0.1	0.89 ± 0.0	0.94 ± 0.1	0.97 ± 0.1	0.64
DFR (% of BW)	1.7^{a} ± 0.1	1.5^{ab} ± 0.1	1.6^{ab} ± 0.1	0.9^{b} ± 0.1	1.2^{ab} ± 0.1	1.5^{ab} ± 0.1	1.2^{ab} ± 0.1	1.4^{ab} ± 0.4	<0.01
RFI (% of BW day^{-1})	1.1^{ab} ± 0.0	1.1^{ab} ± 0.0	1.2^{a} ± 0.0	0.9^{b} ± 0.0	1.0^{ab} ± 0.0	1.1^{ab} ± 0.0	1.0^{ab} ± 0.0	0.9^{b} ± 0.1	0.01
Relative body indices									
HSI (%)	1.71^{ab} ± 0.08	1.68^{ab} ± 0.02	1.43^{ab} ± 0.02	1.42^{ab} ± 0.10	1.59^{ab} ± 0.07	1.76^{a} ± 0.08	1.38^{b} ± 0.06	1.55^{ab} ± 0.10	0.01
VSI (%)	9.96 ± 0.37	9.88 ± 0.32	9.67 ± 0.34	9.91 ± 0.41	10.69 ± 0.54	10.04 ± 0.29	9.5 ± 0.34	10.74 ± 0.06	0.25
Nutrient retention (%)									
Protein (Nx6.25)	49.1 ± 1.4	49.3 ± 0.7	44.3 ± 2.1	44.9 ± 1.1	43.6 ± 2.8	50.3 ± 3.7	44.3 ± 0.8	42.9 ± 2.9	0.18
Crude lipids	88.3 ± 4.8	86.7 ± 1.9	78.4 ± 4.1	77.14 ± 2.0	82.6 ± 0.8	87.4 ± 8.4	79.1 ± 3.2	82.0± 1.1	0.56
Gross Energy	45.3 ± 2.3	47.3 ± 0.7	41.1 ± 3.3	39.2 ± 1.8	40.4 ± 3.1	46.2 ± 4.9	41.0 ± 3.7	38.0 ± 0.5	0.19
Phosphorus	44.2 ± 3.8	46.6± 5.0	39.6 ± 2.4	52.7 ± 2.6	39.5 ± 3.9	36.8 ± 2.2	44.3 ± 1.0	42.2 ± 5.2	0.06
Whole body composition[a]									

Protein (g kg^{-1})	169.1 ± 0.4	170.1 ± 0.7	169.9 ± 2.3	174.6 ± 3.4	170.3 ± 1.4	169.9 ± 0.9	169.8 ± 1.64	171.1 ± 1.9	0.43
Crude lipids (g kg^{-1})	116.1^{a} ± 2.3	115.4^{a} ± 2.9	115.8^{a} ± 3.1	98.4^{b} ± 2.3	103.9^{ab} ± 2.9	114.1^{ab} ± 4.8	104.0^{ab} ± 2.23	102.2^{ab} ± 5.6	0.04
Gross energy (MJ kg^{-1})	8.6 ±0.1	8.7 ± 0.1	8.5 ± 0.3	8.0 ± 0.2	8.2 ± 0.1	8.5 ± 0.3	8.2 ± 0.32	8.1 ± 0.1	0.30
Ash (g kg^{-1})	24.5 ± 0.7	24.5 ± 0.5	23.6 ± 0.9	24.9 ± 0.2	23.8 ± 1.2	24.8 ± 0.3	23.3 ± 0.27	26.4 ± 0.6	0.06
Intestinal morphology[3]									
Mucosal fold height (μm)	293 ± 11.5	270 ± 15.4	267 ± 19.0	303 ± 5.8	299 ± 25.1	289 ± 11.8	272 ± 16.3	300 ± 22.4	0.43
Mucosal fold width (μm)	10.37 ± 0.7^{a}	10.29 ± 0.6^{a}	11.54 ± 0.7^{ab}	15.67 ± 1.3^{b}	12.10 ± 1.5^{ab}	10.30 ± 0.9^{a}	9.60 ± 1.6^{a}	9.93 ± 1.1^{a}	0.01
Goblet cells mm^{-1}	55 ± 5.4	53 ± 4.1	46 ± 2.4	41 ± 4.7	46 ± 4. 1	38 ± 4.9	40 ± 4.6	40 ± 1.7	0.74

[1]Fish meal-based reference diet (FM) and diets with 20%, 40% and 60% fish meal protein replaced with *Saccharomyces cerevisiae* (S20, S40, S60) or a 70:30 mix (biomass ratio) of *Wickerhamomyces anomalus* and *S. cerevisiae* (W20, W40, W60), with an additional *S. cerevisiae* diet without methionine supplementation (S60-Met).

[2]n= 3. Values within rows with different superscripts are significantly different ($P<0.05$).

[3]n=8-12

[a]Whole body composition of the fish sampled before the experiment (initial sample): 164.7 g kg^{-1} crude protein, 65.8 g kg^{-1} crude lipids, 7.98 MJ kg^{-1} gross energy, 25.0 g kg^{-1} ash

Table 5. Apparent digestibility coefficient (ADC; %) for dry matter (DM), crude protein (CP), phosphorus and indispensable amino acids (IAA) and the feed recovery (FR%) of the experimental diets for rainbow trout, n=3. Data presented are least square means ± standard deviation.

	Diet[1]								
	FM	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**	**P-value**
DM	74.94 ± 0.22^{a}	73.44 ± 0.49^{ab}	73.57 ± 0.62^{ab}	72.75 ± 0.45^{ab}	72.31 ±0.61^{b}	74.66 ± 0.17^{ab}	72.47 ± 0.36^{ab}	72.86 ± 0.86^{ab}	0.04
CP	91.23 ± 0.12^{a}	87.67 ± 0.41^{bc}	86.77 ± 0.82^{bc}	86.33 ± 0.77^{c}	86.10 ± 0.87^{c}	89.53 ± 0.27^{ab}	88.13^{bc} ±0.17	86.97 ± 0.60^{bc}	<.0001
Phosphorus	65.23 ±1.69^{a}	69.66 ± 0.87^{ab}	67.43 ± 2.52^{ab}	71.40 ± 1.13^{ab}	70.83 ± 2.63^{ab}	75.10 ± 0.15^{b}	71.43 ± 1.07^{ab}	68.73 ± 2.34^{ab}	0.04
IAA									
Arginine	96.01 ± 0.24^{a}	93.02 ± 0.13^{bc}	91.64 ± 0.78^{bc}	90.59 ± 0.85^{c}	91.21 ± 0.88^{cd}	94.02 ± 0.12^{ab}	93.24 ± 0.11^{bd}	91.96 ± 0.16^{b}	<.0001
Histidine	93.83 ± 0.22^{a}	89.73 ± 0.22^{bc}	88.78 ± 0.48^{c}	88.01 ± 0.85^{c}	88.26 ± 0.83^{c}	91.58 ± 0.08^{ab}	90.21 ± 0.30^{bc}	89.04 ± 0.19^{c}	<.0001
Isoleucine	94.69 ± 0.21^{a}	89.89 ± 0.44^{bd}	87.61 ± 1.00^{cd}	85.71 ± 1.24^{c}	86.37 ± 1.16^{cd}	91.60 ± 0.25^{ab}	89.67 ± 0.09^{bd}	87.04 ± 0.14^{cd}	<.0001
Leucine	95.32 ± 0.16^{a}	91.45 ± 0.31^{bc}	89.41 ± 0.87^{cd}	87.59 ± 1.08^{d}	88.35 ± 0.93^{d}	93.05 ± 0.21^{ab}	91.58 ± 0.13^{bc}	89.33 ± 0.12^{cd}	<.0001
Lysine	94.47 ± 0.19^{a}	89.80 ± 0.36^{bc}	88.31 ± 1.00^{bc}	86.83 ± 1.26^{c}	87.27 ± 1.21^{c}	91.75 ± 0.19^{ab}	90.77 ± 0.09^{bc}	88.30 ± 0.18^{bc}	<.0001
Methionine	95.14 ± 0.26^{a}	93.96 ± 0.21^{ab}	92.99 ± 0.55^{ab}	92.37 ± 0.69^{b}	89.12 ± 0.92	94.91 ± 0.11^{a}	94.47 ± 0.17^{ab}	93.44 ± 0.07^{ab}	<.0001
Phenylalanine	95.81 ± 0.28^{a}	92.63 ± 0.29^{bcd}	91.02 ± 0.73^{b}	89.88 ± 0.91^{d}	90.24 ± 1.04^{cd}	93.53 ± 0.39^{ab}	93.28 ± 0.63^{abc}	90.56 ± 0.07^{b}	<.0001
Threonine	92.16 ± 0.38^{a}	85.53 ± 0.40^{b}	83.59 ± 0.84^{bc}	79.99 ± 1.10^{d}	81.27 ± 1.30^{cd}	87.56 ± 0.15^{b}	85.44 ± 0.42^{b}	82.38 ± 0.18^{bcd}	<.0001
Valine	94.55 ± 0.22^{a}	89.66 ± 0.38^{b}	87.51 ± 0.95^{cd}	85.58 ± 1.22^{d}	86.32 ± 1.15^{cd}	91.27 ± 0.23^{ab}	89.51 ± 0.16^{b}	87.25 ± 0.17^{cd}	<.0001
Sum of IAA	94.46 ± 0.20^{a}	91.04 ± 0.27^{bcd}	89.87 ± 0.65^{b}	88.52 ±0.87^{d}	88.90 ± 0.92^{d}	92.41 ± 0.14^{ab}	91.57 ± 0.15^{bc}	89.48 ± 0.16^{c}	<.0001
FR (%)*	65.02±3.59^{b}	85.65±0.44^{a}	61.78±0.29^{b}	53.77±1.62^{bc}	51.42±2.82^{bc}	58.28±1.71^{b}	40.60±6.76^{c}	42.46±3.11^{c}	<.0001

[1]Fish meal-based reference diet (FM) and diets with 20%, 40% and 60% fish meal protein replaced with *Saccharomyces cerevisiae* (S20, S40, S60) or a 70:30 mix (biomass ratio) of *Wickerhamomyces anomalus* and *S. cerevisiae* (W20, W40, W60), with an additional *S. cerevisiae* diet without methionine supplementation (S60-Met). * n=2

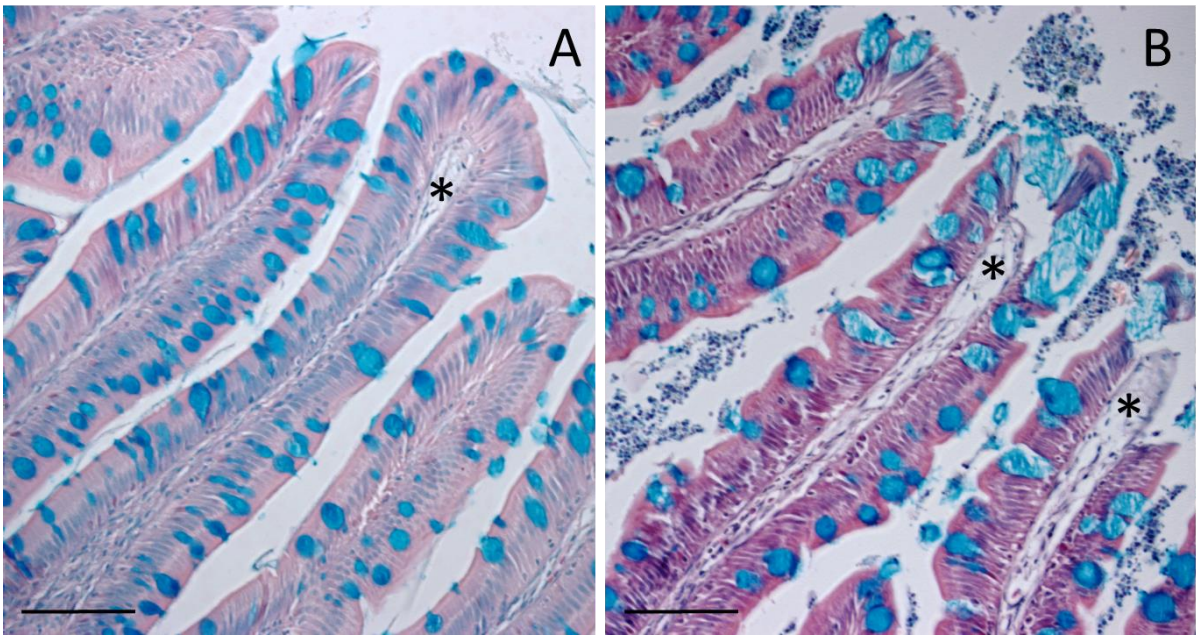

Fig 1.

Sections from proximal intestine stained with haematoxylin and eosin/alcian blue stain (pH 2.5). Oedematous mucosal fold tips (*) were visible in the FM diet group (A), but the oedema was enhanced in the S60 diet group (B). Scale bar represents 100 μm.